The KEN box regulates Clb2 proteolysis in G1 and at the metaphase-to-anaphase transition

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Clb2 mitotic cyclin inhibits cell cycle progression by preventing mitotic exit and DNA synthesis. To allow cell cycle progression, Clb2 proteolysis is triggered by Cdc20 during the metaphase-to-anaphase (M-A) transition and by Hct1 during mitotic exit and G1 [1–6]. A *cis* element called the destruction box is required for this proteolysis [7–11]. Recently, an additional *cis* element called the "KEN box" was also shown to be required for proteolysis of human CDC20 and Securin [3, 12]. Using a novel color assay, we show that a Clb2 KEN box is required to target a fusion protein containing the first 124 amino acids of Clb2 for proteolysis. We further show that full-length Clb2 bearing mutations in the KEN box is degraded efficiently during the M-A transition, but poorly during G1. If the destruction box of Clb2 is mutated in combination with mutation of the KEN box, then this form of Clb2 is more stable than Clb2 bearing either mutation by itself during both M-A and G1. Our results show that the KEN box and the destruction box act together during both M-A and G1 to regulate Clb2 proteolysis.

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Received: **22 June 2001**
Revised: **18 September 2001**
Accepted: **18 September 2001**

Published: **13 November 2001**

Current Biology 2001, 11:1781–1787

0960-9822/01/$ – see front matter

Results and discussion

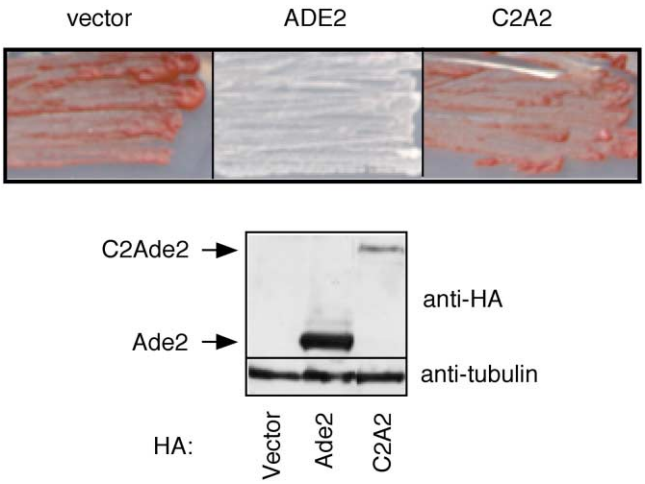
Protein degradation within a cell must be tightly regulated. To ensure that degradation remains specific to a subset of cellular proteins, proteolytic pathways require that targeted proteins contain specific *cis*-acting motifs. To assist in the identification and characterization of these motifs, we have used an in vivo protein stability assay that we developed in *Saccharomyces cerevisiae*. This assay allows us to rapidly screen protein-coding sequences for *cis* elements that affect their abundance.

Our assay utilizes two well-studied biochemical properties. First, if a protein normally targeted for ubiquitin-mediated proteolysis is fused to a stable protein, then the fusion protein is targeted for ubiquitin-mediated proteolysis [13]. Second, *S. cerevisiae ade2* strains deficient for the *ADE2* gene product accumulate a red adenine pathway intermediate when this pathway is induced. Thus, when these strains are grown on plates containing limited adenine, they form red colonies [14]. We therefore reasoned that the fusion of a gene encoding an unstable protein to *ADE2* in an *ade2* mutant should mimic the red colony color phenotype of an *ade2* mutant.

We tested this hypothesis by fusing a gene encoding a known unstable protein, the mitotic cyclin *CLB2*, to *ADE2* in a CEN/ARS plasmid (Figure 1). An *ade2-101* mutant strain, transformed with empty vector, produced colony patches with a deep red color. As expected, the same mutants transformed with a vector containing *ADE2* expressed constitutively from the *ADH1* promoter formed white colony patches. We next fused the gene encoding Clb2 in frame with the *ADE2* gene and transformed this construct into the *ade2* mutant strain. The resulting colonies were red, suggesting that the fusion protein was of low abundance, possibly because it was being targeted for ubiquitin-mediated proteolysis by Clb2 motifs (Figure 1). To ensure that the change in colony color reflected a true decrease in protein content, we performed Western analysis on cells taken directly from the plate colonies to examine protein levels. The results of this analysis showed that Ade2 alone was more abundant than the C2Ade2 fusion protein (Figure 1). Thus, the degree of color change from red to white parallels the relative abundance of the C2Ade2 or Ade2 proteins. As an additional control, we tested whether fusion of a stable protein, GFP, affected Ade2 stability. Fusion of the gene encoding *GFP* to *ADE2* resulted in similar levels of GFPAde2 and Ade2, as reflected by either colony color or protein level (Figure 2b, GFP). These results encouraged us to use this color assay to characterize sequences within Clb2 that target the protein for degradation.

The N terminus of some B-type cyclins is sufficient to target them for ubiquitin-mediated proteolysis. For example, the destruction box in the N terminus of *Xenopus* cyclin B1 and sea urchin cyclin B is sufficient to allow ubiquitin to be conjugated onto adjacent lysines and to target the protein for proteolysis [11, 15, 16]. In contrast, fragments of *Xenopus* cyclin A or B2 that are of similar

Figure 1



Development of a protein stability color assay. S288c strains maintaining a vector bearing *ADH1* promoter-driven expression of either no insert (vector), *ADE2HA* (ADE2), or *CLB2* fused to *ADE2HA* (C2A2) were plated on SD minimal medium containing a limiting concentration of adenine (6 μg/ml). The concentration of adenine used was sufficient for the growth of *ade2* strains, yet was low enough to activate the adenine biosynthetic pathway, allowing for production of the red intermediate. Plates were incubated for 96 hr at 30°C to allow sufficient time for the development of colony color. To confirm the correlation of color in the plate assay with cellular protein levels, cells were scraped directly from plates, washed once with water, and prepared for Western analysis. Protein extracts were transferred to nitrocellulose and probed with either anti-HA, to detect Ade2 levels, or anti-α tubulin, to control for sample loading.

size and bear similar motifs are not degraded unless additional *cis* elements are added [11].

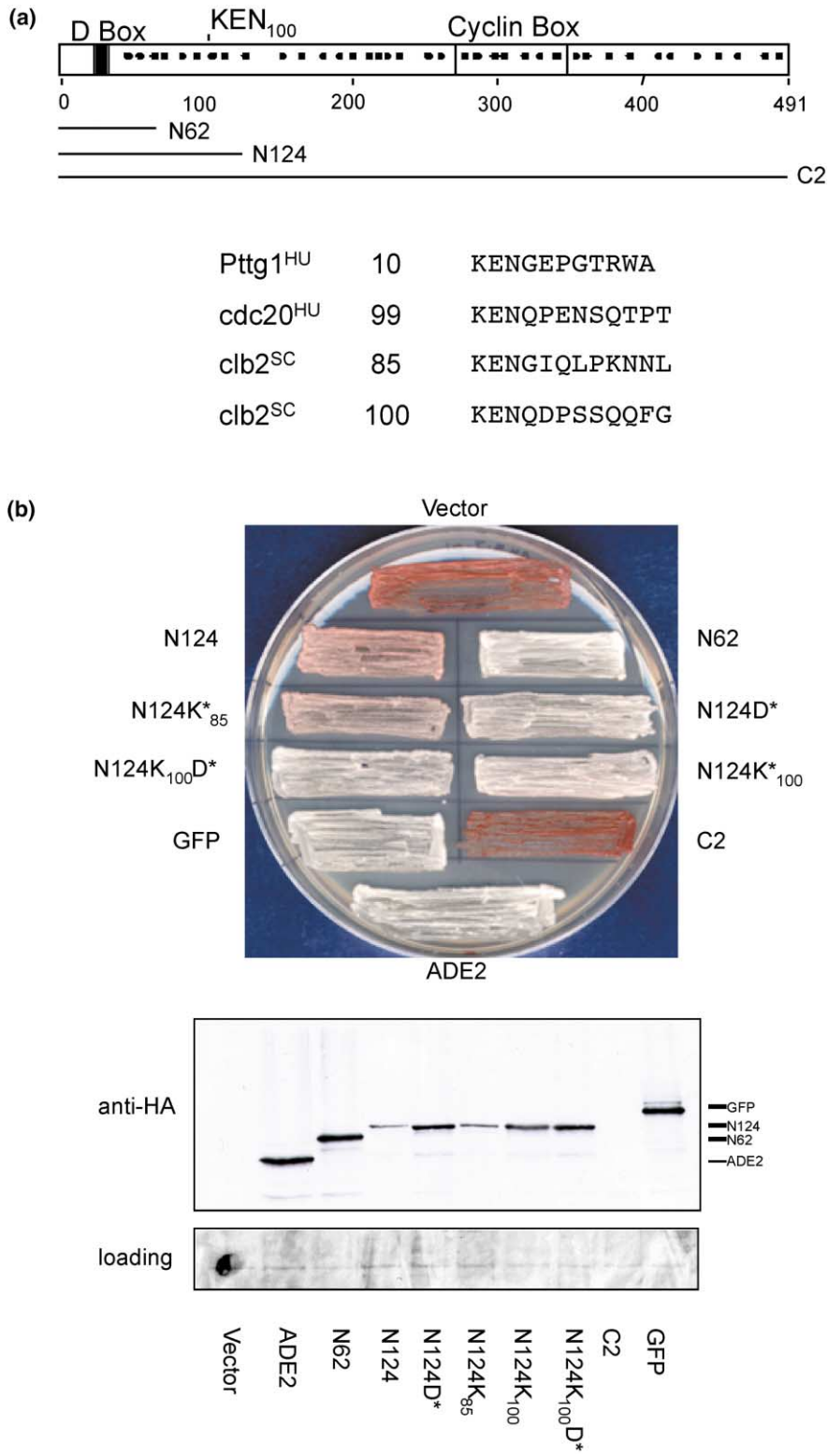
We tested the N terminus of Clb2 to determine whether an N-terminal fragment containing the destruction box was sufficient to destabilize Ade2. We used our color assay to test the stability of a 62 amino acid N-terminal fragment of yeast mitotic cyclin Clb2, which included the destruction box and four lysines, fused to Ade2 (N62) (Figure 2a,b). *ade2* mutants expressing N62 from the *ADH1* promoter on a CEN/ARS plasmid gave rise to white colonies (Figure 2b). This suggested that the N62 fusion protein with Ade2 was present at similar levels to the Ade2 or GFPAde2 control proteins. This was confirmed by Western analysis of N62 and GFP fusion protein and Ade2 protein in these cells (Figure 2b). Thus, the N terminus of Clb2 containing the destruction box was insufficient to efficiently target this Ade2 fusion for degradation. This suggested that the presence of additional motifs within Clb2 contribute to the destabilization of the Ade2 fusion.

A KEN box is required for ubiquitin-mediated proteolysis of human Cdc20 and Securin [3, 12]. Close examination of the Clb2 ORF revealed two putative KEN boxes at amino acid positions 85 and 100 (Figure 2a). We tested the ability of the KEN boxes of CLB2 to target the protein for proteolysis. To do this, we fused the first 124 amino acids of Clb2, containing the destruction box and the KEN boxes, to Ade2 (N124) and repeated the color assay. Transformation of this construct into *ade2* mutants gave rise to colonies that were more pink than *ade2* mutants transformed with N62, although not as red as strains transformed with vector alone or with full-length Clb2 fused to Ade2. Extracts made from these cells confirmed the presence of less N124 protein fusion than strains expressing the N62 or GFP protein fusions or ADE2 alone, but more than strains expressing Clb2 fused to Ade2 (Figure 2b).

We investigated the role of the KEN boxes in Clb2 proteolysis, both alone and in conjunction with the destruction box, by creating specific mutations in the two motifs and repeating the color assay. *ade2* mutants expressing the N124K*$_{85}$ fusion construct bearing mutations in the KEN$_{85}$ box were similar in color to strains expressing the N124 fusion construct. In contrast, *ade2* mutants expressing the N124K*$_{100}$ fusion construct with mutations in the KEN$_{100}$ box, or *ade2* mutants bearing constructs expressing the N124D* fusion construct with mutations in the destruction box, formed colonies that were significantly whiter than *ade2* mutants bearing the wild-type N124 fusion construct. Finally, *ade2* mutants expressing N124D*K*$_{100}$ fusion constructs, bearing mutations in both motifs, were also white. Together, these results suggest that both the destruction box and the KEN$_{100}$ box contribute to the proteolytic regulation of Clb2 but that the KEN$_{85}$ box does not. As a final test of the color assay, we used Western analysis to look directly at the quantity of fusion protein in the various colonies. We observed increased levels of all N124 mutant proteins that formed white colonies relative to constructs (N124 and N124K*$_{85}$) that formed pink colonies. Thus, by using the color assay, we identified a functional KEN$_{100}$ box in Clb2 that regulates its abundance and that can act as a transposable degradation element in *S. cerevisia*e.

We pursued the physiological role of the KEN boxes in Clb2 regulation by testing their role in targeting full-length Clb2, not fused to Ade2, for degradation at various stages of the cell cycle. Clb2 proteolysis starts at the metaphase-to-anaphase transition of the cell cycle. This first wave of proteolysis requires Cdc20 protein [1–6]. To test the physiological contribution of the KEN boxes to Clb2 protein stability during this transition, we briefly expressed Clb2 or mutant derivatives lacking the normal destruction box motif, lacking either KEN box motif, or lacking both KEN$_{100}$ and the destruction box motifs from the *GAL1/10* promoter during a metaphase arrest. We then followed the decrease in the levels of Clb2 protein during release from this arrest, as cells progressed from meta-

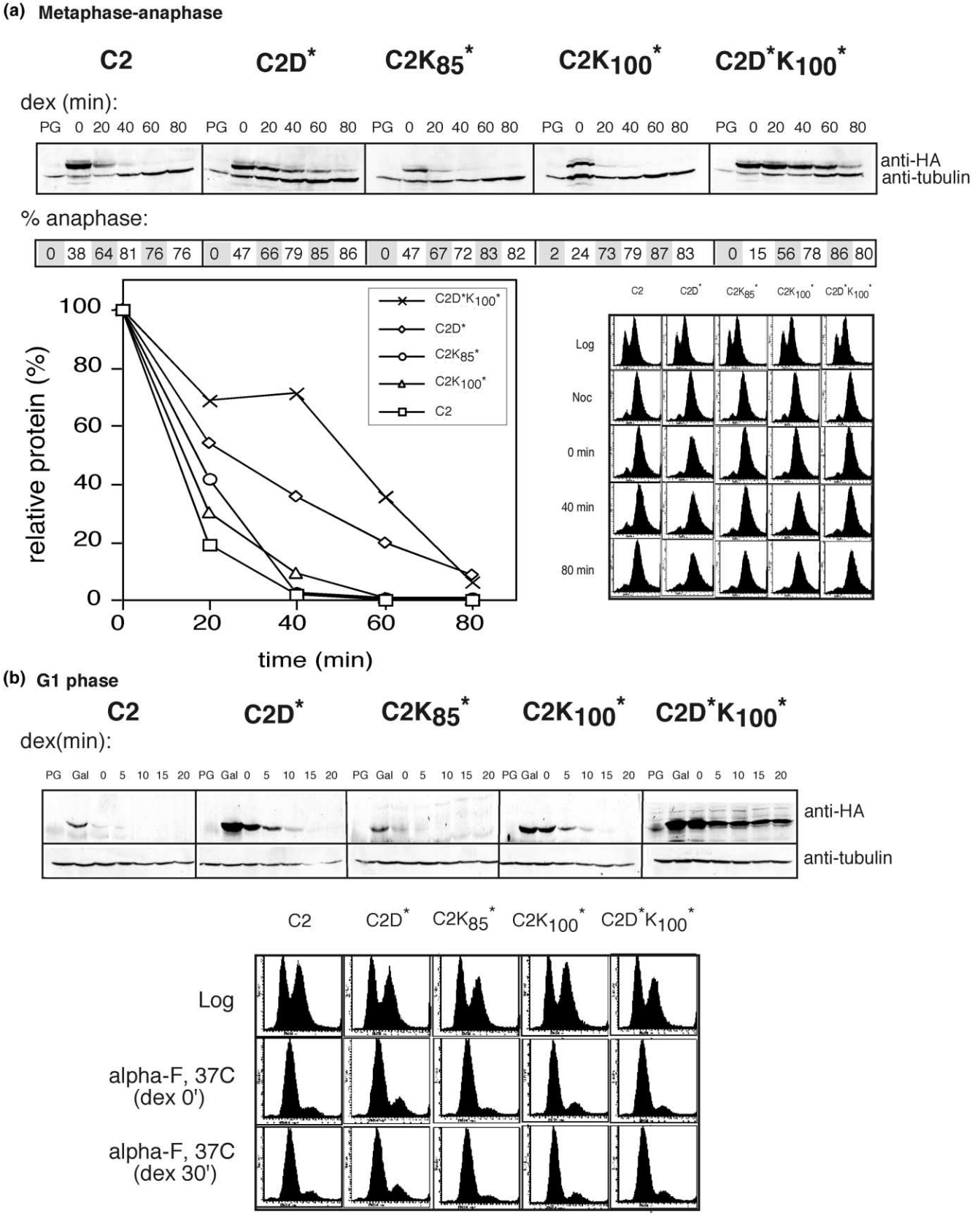
Figure 2



The colony color assay reveals a functional KEN box in *CLB2*. **(a)** A schematic diagram of the Clb2 protein sequence indicating the location of important *cis* elements. CLB2 fragments used are also indicated. D box: destruction box; KEN: KEN box. Dots represent lysine residues. Sequences of two known KEN boxes are aligned with those in Clb2. **(b)** The KEN box and the destruction box are required to destabilize Ade2p. W303 strains bearing vector alone or vector containing *ADH1*-driven expression of: *ADE2HA* (ADE2); the first 62 amino acids of *CLB2* fused to *ADE2HA* (N62); the first 124 amino acids of *CLB2* fused to *ADE2HA* (N124); or mutant versions of N124 with a 9 amino acid deletion of the destruction box (N124D*), N124 bearing the sequence AAA instead of KEN_{85} ($N124K^*_{85}$) or KEN_{100} ($N124K^*_{100}$), or N124 with a destruction box deletion and mutations in the KEN_{100} box ($N124D^*K^*_{100}$) were plated and analyzed for colony color and protein levels on the same plate, as described in Figure 1. C2A2 was observed only after extensive overexposure of the Western blot (data not shown, see Figure 1).

phase to an anaphase arrest imposed by *cdc15-2* (Figure 3a). Wild-type Clb2 protein (C2) and Clb2 with inactivating mutations in the KEN_{85} box ($C2K^*_{85}$) or KEN_{100} box ($C2K^*_{100}$) were rapidly degraded as cells progressed from metaphase to anaphase. Clb2 protein with inactivating mutations in the destruction box (C2D*) was degraded

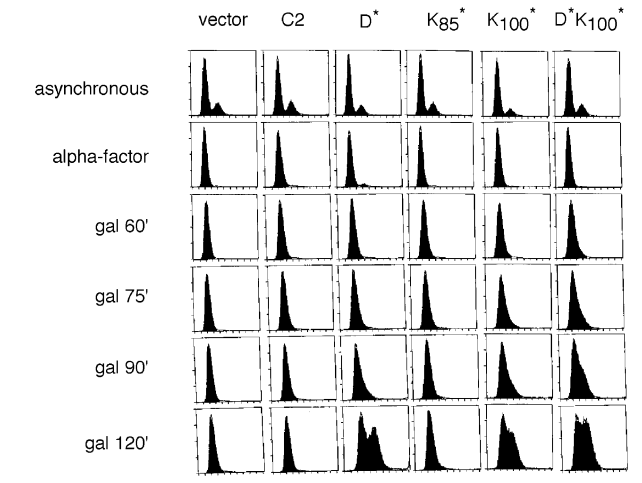
Figure 3


(a) Metaphase-anaphase
C2
C2D*
C2K85*
C2K100*
C2D*K100*
dex (min):
PG 0 20 40 60 80 PG 0 20 40 60 80 PG 0 20 40 60 80 PG 0 20 40 60 80 PG 0 20 40 60 80
anti-HA
anti-tubulin
% anaphase:
0 38 64 81 76 76 0 47 66 79 85 86 0 47 67 72 83 82 2 24 73 79 87 83 0 15 56 78 86 80
relative protein (%)
time (min)
C2D*K100*
C2D*
C2K85*
C2K100*
C2
Log
Noc
0 min
40 min
80 min
(b) G1 phase
C2
C2D*
C2K85*
C2K100*
C2D*K100*
dex(min):
PG Gal 0 5 10 15 20
anti-HA
anti-tubulin
Log
alpha-F, 37C (dex 0')
alpha-F, 37C (dex 30')

much more slowly. Interestingly, Clb2 with inactivating mutations in both the KEN_{100} box and in the destruction box ($C2D^*K^*_{100}$) was degraded even more slowly than Clb2 bearing either mutation alone (Figure 3a). FACS and morphological analysis confirmed that, during this experiment, cells expressing different Clb2 forms remained arrested in mitosis and transited from metaphase to anaphase normally and with similar kinetics, as expected (Figure 3a). Together, these results show that the KEN box acts in conjunction with the destruction box during the metaphase-to-anaphase transition to target Clb2 for ubiquitin-mediated proteolysis.

We next tested the proteolysis of Clb2 during an arrest in G1. Clb2 is normally targeted by Hct1 for proteolysis during this phase of the cell cycle [1–6]. *cdc28-4* mutants transformed with HA-tagged Clb2 constructs (C2, C2D*, $C2K^*_{85}$, $C2K^*_{100}$, $C2D^*K^*_{100}$) were arrested in G1 using α factor and were then shifted to 37°C to inactivate a mutant form of the cyclin-dependent kinase, *cdc28-4*. We utilized the *cdc28-4* mutant to ensure that the expression of Clb2 would not drive the cells into S phase. The G1-arrested cells were exposed briefly to galactose to induce a pulse of Clb2 construct expression (Figure 3b). Under these conditions, C2D*, $C2K^*_{100}$, and $C2D^*K^*_{100}$, but not C2 or $C2K^*_{85}$ protein, accumulated, showing that these latter two proteins were the most unstable of all of the constructs tested (Figure 3b, Gal). Upon promoter shutoff and in the presence of cycloheximide to prevent further translation, C2D* and $C2K^*_{100}$ protein levels dropped, but the $C2D^*K^*_{100}$ protein level dropped significantly more slowly. These results show that, during G1, both the KEN_{100} box and the destruction box contribute to the regulation of Clb2 proteolysis and that the two motifs function additively to regulate Clb2 protein stability. During the course of our experiment, there was no detectable S-phase shift by FACS, and the high (>80%) and constant numbers of unbudded cells indicated effective G1 arrest. Thus, our results were unlikely to be caused by inhibition of Clb2 proteolysis as cells entered S phase (Figure 3b) [17].

Figure 4



Clb2 bearing mutations in the KEN box is functional. The constructs described in Figure 3 were transformed to W303a. The resulting strains were grown in minimal media containing 2% raffinose and were arrested in G1 by the addition of 3 μg/ml α factor for 3 hr. Clb2 expression was then induced from the *GAL1/10* promoter by the addition of galactose to 2%. The effect of Clb2 expression on G1 arrest was monitored by DNA content and cellular morphology.

To ensure that our results were not a consequence of unfolded mutant cyclin proteins, we tested the functionality of our constructs based on the ability of excess Clb2 to cause escape from α factor arrest (Figure 4) [17]. Yeast were arrested and maintained in G1 with α factor (but with functional Cdc28 protein). Then Clb2, or the mutant derivatives, was expressed from the *GAL1/10* promoter in wild-type cells, and the response of cells was monitored by

The KEN box and destruction box function additively to regulate Clb2 degradation. **(a)** Clb2 proteolysis during the metaphase-to-anaphase transition. Full-length wild-type *CLB2* (C2) or *CLB2* containing mutations in the destruction box (C2D*), in either KEN box ($C2K^*_{85}$ and $C2K^*_{100}$), or in the destruction box and in the KEN_{100} box ($C2D^*K^*_{100}$) were cloned behind the *GAL1/10* promoter and were transformed into a *cdc15-2* mutant strain. Pulse-chase analysis of protein levels was performed exactly as described by Baumer [1]. Briefly, the strains were pregrown in minimal media with 2% raffinose to mid-log phase, collected, and resuspended in rich media with 2% raffinose containing 15 ug/ml nocodazole to arrest the cells at metaphase. Clb2 expression was then induced for 1 hr by adding galactose to 2%, and Cdc15 was inactivated by a temperature shift to 35°C for the last 30 min of galactose treatment. Clb2 expression was then turned off by adding dextrose to 2%, and cells were washed and suspended in fresh media at 37°C to release them from their metaphase arrest and further inactivate Cdc15 and were kept at 37°C. Using this protocol, time 0 is approximately 30 min after nocodazole removal, as cells progress to anaphase (defined as: large budded cells, two nuclei, no new buds). Western analysis was performed for Clb2 levels (anti-HA) and sample loading (anti-α tubulin). Samples were also analyzed for DNA content by flow cytometry and were analyzed for cell cycle stage by microscopy. PG; samples taken prior to galactose induction. 0, 20, 40, 60, and 80 min; samples taken after nocodazole washout and as cells progress to anaphase. **(b)** Clb2 proteolysis during G1. *cdc28-4* mutants transformed with the same plasmids as in (a) were grown in minimal media plus 2% raffinose and were then arrested in G1 by treatment with 3 μg/ml α factor for 3 hr. Cells were then resuspended in rich media plus raffinose prewarmed to 37°C, to inactivate the Cdc28 kinase, plus 3 μg/ml α factor. During this G1 arrest, a pulse of Clb2 expression was induced by adding galactose (2%) for 1 hr followed by the addition of dextrose (2%) and cycloheximide (50 μg/ml). PG; samples of cells taken prior to galactose addition. 0, 5, 10, 15, and 20 min; samples taken after dextrose and cycloheximide addition. Samples were analyzed as in (a).

FACS (Figure 4). Overexpression of Clb2, or its stabilized derivatives, bypassed the α factor arrest, in a fashion that paralleled the stability of the Clb2 protein. None of the cultures budded during the course of the experiment, suggesting that Cln kinase levels remained low. Thus, the constructs were functional by this assay. In addition, during our analysis we found that stabilized Clb2 derivatives induced aneuploidy in *cdc15-2* mutants (but not in wild-type cells) at the permissive temperature, in a fashion that paralleled their stability (data not shown). The nature of this genetic interaction between Clb2 and *cdc15* mutants is not clear.

We have developed a colony color assay that can be used to rapidly identify *cis* elements within proteins, which regulate their abundance in yeast. This assay is useful because, in addition to the application presented, it can be used in screens to identify genes required in *trans* for the proteolysis of test substrates. In addition, it can be used to test the effect of particular treatments (drugs, DNA damage) on substrate proteolysis.

Using this assay, we have characterized a functional KEN box motif in *S. cerevisiae*, located in the Clb2 mitotic cyclin. We have shown that KEN_{100} functions together with the destruction box to target Clb2 for ubiquitin-mediated proteolysis during the M-A transition, but especially during G1. Our color assay results suggest that Clb2, like *Xenopus* cyclins A and B2 which also require the cyclin box, may also require additional sequences for proteolysis.

In *S. cerevisiae*, Cdc20 and Hct1 activity is regulated in a manner that ensures that Cdc20 acts first, at the metaphase-to-anaphase transition, and Hct1 acts next, during G1, to trigger Clb2 proteolysis [1–6]. Our in vivo results showing that the KEN box is important for Clb2 proteolysis during both phases of the cell cycle suggest that Cdc20/APC/C and Hct1/APC/C both interact with the KEN box sequence of Clb2. One model to explain why the KEN and destruction boxes play additive roles in Clb2 proteolysis is that they function together, especially during G1, to allow proper orientation of the substrate on the APC/C prior to its ubiquitination.

Materials and methods

Strains and plasmids

The yeast strains used in this study were S288c (*MATa ura3-52 leu2Δ1 his3Δ 200 trp1Δ 63 lys2-801 ade2-101*) and W303a (*MATa ura3 leu2-2,112 his3-11 trp1-1 ade2-1 can1-100 GAL [psi+]*). The *cdc28-4* and *cdc15-2* strains areW303a derivatives. All yeast protocols were performed by standard procedure [18].

The plasmids generated for this study were all derivatives of the *URA3 CEN/ARS* plasmid pRS416 [19]. This vector was modified for protein expression by insertion of the *ADH1* promoter into NotI/EcoRI sites and by insertion of the *ADH1* transcriptional terminator into XhoI/BglII sites. For the *ADE2* fusion plasmids, the *ADE2* ORF was PCR amplified and cloned into EcoRI/ClaI sites of the expression-modified pRS416. Using this base fusion plasmid, we were able to insert truncated and/or mutated *CLB2* ORFs via the EcoRI restriction site. Plasmids used for galactose induction studies were created by swapping the *ADH*1 promoter in our *ADH1-CLB2* plasmids with the *GAL1/10* promoter from a SphI/SpeI fragment of *pGAL-CLB2* [20]. All constructs were tagged with a triple HA epitope. Details of vector construction and primers used are available upon request.

DNA content analysis

Cells were prepared for analysis of DNA content by flow cytometry as described using a Coulter Epics Elite ESP flow cytometer [21].

Western analysis

Western analysis was performed by standard techniques [22]. Antibodies used were mouse HA.11 (Covance Research Products) and rat anti-tubulin (Harlan Sera-Lab). Analysis was performed by HRP-conjugated secondary antibody using ECL detection (Amersham Pharmacia Biotech).

Acknowledgements

We would like to thank Angelika Amon, Steve Kron, Kim Nasmyth, Orna Cohen-Fix, and David Roof for strains, plasmids, and antibodies and Dave Dicker for flow cytometry. This work was supported by the Howard Hughes Medical Institute and by a training grant from the National Institutes of Health to C.H. (T36-GM08216). We would also like to thank the members of the Holloway lab for helpful discussions.

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